# A differential response element for the homeotics at the Antennapedia P1 promoter of *Drosophila*

(transcription/gene regulation/homeodomain protein)

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ABSTRACT Homeotic genes encode DNA-binding tran-
scription factors that specify the identity of a segment or
segments in particular body regions of *Drosophila*. The devel-
opmental specificity of these proteins results from their differ-
ential regulation of various target genes. This specificity could
be achieved by use of different regulatory elements by the
homeoproteins or by use of the same elements in different ways.
The Ultrabithorax (UBX), abdominal-A (ABD-A), and Anten-
napedia (ANTP) homeoproteins differentially regulate the An-
tennapedia P1 promoter in a cell culture cotransfection assay:
UBX and ABD-A repress, whereas ANTP activates P1. Either
of two regions of P1 can confer this pattern of differential
regulation. One of the regions lies downstream and contains
homeoprotein-binding sites flanking a 37-bp region called
BetBS. ANTP protein activates transcription through the bind-
ing sites, whereas UBX and ABD-A both activate transcription
through BetBS and use the flanking binding sites to prevent this
effect. Thus, homeoproteins can use the same regulatory
element but in very different ways. Chimeric UBX–ANTP
proteins and UBX deletion derivatives demonstrate that func-
tional specificity in P1 regulation is dictated mainly by se-
quences outside the homeodomain, with important determi-
nants in the N-terminal region of the proteins.

The homeotic selector genes that include Antennapedia
(*Antp*), Ultrabithorax (*Ubx*), and abdominal-A (*abd-A*) are a
family of related but distinct developmental regulators that
specify the differences in the body segments of *Drosophila
melanogaster* (1–3). Each gene and the homeoproteins it
encodes (ANTP, UBX, and ABD-A, respectively) is associ-
ated with the identity of a particular segment or group of
segments in which it is expressed. The homeotics are similar
in that they act at similar times and control homologous
aspects of morphology in the different segments. In certain
cells and tissues different homeotics even have identical
functions. For example, *Ubx* and *abd-A* each suppress the
formation of sensory structures called Keilin's organs in their
respective domains of expression (1). But these regulators are
also quite distinct from one another, as seen by the different
segment morphologies that result when they are artificially
expressed in the same domain during development (4–6).

The ANTP, UBX, and ABD-A homeoproteins are DNA-
binding transcription factors that show 90% or more identity
in their DNA-binding homeodomains but show very little
similarity outside this 60-residue region (7, 8). Studies of
homeoproteins *in vitro* demonstrate that their intrinsic DNA-
binding specificities are very similar (9–13), and they can act
through the same sites and regulate the same target genes in
cell culture cotransfection experiments (10, 14–16) and prob-
ably in the developing animal as well (11, 13). These findings
can explain their common developmental functions.

The distinct developmental functions of the homeoproteins
presumably result from differences in target gene regulation:
either they regulate different target genes or they regulate the
same target genes differently. Such regulatory differences
could be due to use of different regulatory elements (*target-
ing* differences), or from differences in the functional conse-
quences of the proteins at the same regulatory elements
(*activity* differences). Although their similar intrinsic DNA-
binding properties argue for the importance of activity dif-
ferences, some differences in binding specificity *in vitro* have
been detected (12, 13, 17). Furthermore, targeting *in vivo*
might be altered in a variety of ways, such as by interactions
with coregulators, as shown for some distantly related ho-
meodomain proteins (18, 19). Most studies of chimeric ho-
meoproteins indicate that developmental specificity is dic-
tated by structural elements in or near the homeodomain (5,
20–24), but since this region may harbor activity determi-
nants as well as DNA-binding and targeting determinants,
these results do not unambiguously distinguish between the
specificity mechanisms. The mechanisms are best distin-
guished by precise mapping of homeoprotein response ele-
ments at genes that are differentially regulated by the ho-
meoproteins.

*Antp* is itself differentially regulated by the homeotics. Its
two promoters, P1 and P2, are separated by ≈65 kb, and they
are under independent control; both are repressed by UBX
and ABD-A in many tissues where expression of the homeot-
ics overlaps (25–28). In contrast, ANTP has little effect on its
own expression in most tissues (26, 28), and where effects
have been observed they are stimulatory (13). The homeopro-
teins also differentially regulate P1 in a cotransfection assay
in *Drosophila* S2 cells: UBX and ABD-A repress P1, whereas
ANTP weakly activates P1 (refs. 10 and 14; B. Appel, S.
Sakonju, and M.A.K., unpublished data; see below). Two
UBX response elements have been identified in this assay (E.
Parker and M.A.K., unpublished data). The major element
maps to the P1 core promoter region, away from all high-
affinity UBX-binding sites. UBX represses through this
element by somehow rendering the promoter insensitive to
upstream enhancers. A second element lies downstream and
includes two UBX-binding regions centered at +320 and
+420 bp.

In this paper, we map the ABD-A and ANTP response
elements at P1 and localize structural determinants in the
homeoproteins that confer specificity in regulation of P1. We
find that the three homeoproteins can all act through the same
downstream element to differentially regulate P1 expression,
demonstrating that regulatory specificity can result from
differences in activity rather than targeting. At least part of
this specificity stems from differences in their interactions

with an unusual regulatory element in the 37-bp region that separates the two UBX-binding regions.

## MATERIALS AND METHODS

**Transcriptional Reporter Plasmids.** The pP$_{AntpP1}$CAT reporter plasmid contains a genomic fragment of P$_{AntpP1}$ (−6 to +0.79 kb) in pC4CAT (14). The other plasmids in Fig. 1 (pEP1015, pEP1006, and pEP1009) are deletion derivatives of pP$_{AntpP1}$CAT made by E. Parker (E. Parker and M.A.K., unpublished work). ΔAB (pES2000) has the A and B binding regions in the −0.42- to +0.79-kb *Antp* P1 construct replaced with *Nhe* I and *Spe* I restriction sites, respectively. ΔAB/BetBS (pES2005) was made by cutting ΔAB with *Nhe* I and *Spe* I and religating to remove the intervening BetBS region. Constructs with the A (5′-GCCCGCTAAGATCATAAAG-GCCGTAAAAATAATCATAACAATAATCG-TAAAAAATTAGAACCG) and B (5′-AGATCGTCAT-AAACCCATTATTATAATAATAACCGTAATCGTAAC-CGTAATCGTAATTGTAATCGCAG) UBX-binding regions (+290 to +352 and +385 to +452 of P1, respectively) inserted at −47 bp at the *Adh* (alcohol dehydrogenase) distal promoter were made by cloning these sequences with added *Xba* I linkers into the *Xba* I site of pD-33CAT.

**Homeoprotein Expressor Plasmids and Transfections.** Expressor plasmids have cDNAs that encode the homeoproteins inserted downstream of the actin *5C* promoter in the vector pPac (14). pPacUBX Ia$^{wt}$, pPacUBX Ib$^{S/B}$ (which has a frameshift mutation in *Ubx* codon 8), and pPacANTP Ib are described elsewhere (10, 14, 16). pPacABD-A (provided by B. Appel and S. Sakonju, University of Utah) has a 1.9-kb *abd-A* cDNA inserted in the *Bam*HI site of pPac. pPacABD-A$^{fs}$ has a frameshift mutation in *abd-A* codon 16 that was
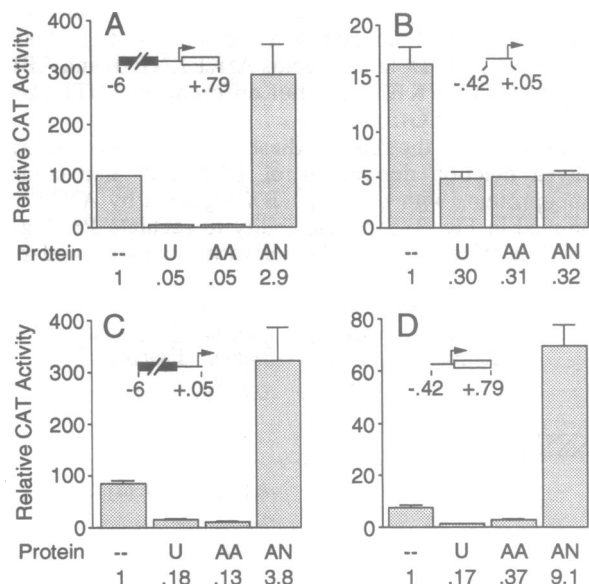


FIG. 1. Effects of UBX, ABD-A, and ANTP homeoproteins on deletion derivatives of the *Antp* P1 promoter. *Drosophila* S2 cells were cotransfected with various P1 promoter constructs driving expression of chloramphenicol acetyltransferase (CAT) and either a UBX (U, pPacUBX Ia$^{wt}$), an ABD-A (AA, pPacABD-A), an ANTP (AN, pPacANTP Ib) expressor plasmid, or a nonexpressing control plasmid (−, pPacUBXIb$^{S/B}$) as indicated. The P1 constructs tested were as follows: (*A*) pP$_{AntpP1}$CAT, (*B*) pEP1015, (*C*) pEP1009, and (*D*) pEP1006. Their structures are schematized; the filled and open boxes represent the two regions of P1 that can confer a differential response to the homeotics. CAT activity in extracts of the cotransfected cells is shown, normalized to the basal activity of the standard P1 construct in *A*. Values are the average and standard error of at least two experiments, each done in duplicate. Numbers below each graph show the relative effects of the homeoproteins.

introduced by cutting pPacABD-A with *Bsp*EI and filling-in the ends. Plasmids that express UBX deletion derivatives and chimeric UBX–ANTP proteins were constructed in pPac from related constructs in the CaSpeR vector (23). For pPacUAU and pPacUAΔ, a 2.2- or 2.0-kb *Bam*HI–*Eco*RI fragment, respectively, from the related CaSpeR construct, was end-filled and inserted into the filled *Bam*HI site of pPac. For pPacUUΔ, a 2-kb *Bam*HI fragment was inserted into the *Bam*HI site of pPac. For pPacUAA, a 1.8-kb *Stu* I–*Eco*RI fragment was end-filled and used to replace the *Stu* I/*Xba* I fragment containing almost the entire *Ubx* coding and 3′ untranslated regions of a plasmid (pPacUBX$^{Q50A/N51A/R53A}$) that contains an introduced *Xba* I site in the 3′ untranslated region to facilitate cloning. These plasmids produced similar levels of proteins (within 2-fold of wild-type UBX), as determined by immunoblotting of S2 cell extracts with a UBX antibody. The apparent molecular mass of the proteins were as follows: UBX Ia, 37 kDa (calculated mass 39.1 kDa); UAA, 32 kDa (37.8 kDa); UAU, 37 kDa (39.1 kDa). UUΔ and UAΔ each produced two approximately equimolar species with a molecular mass of 29 and 32 kDa (calculated mass 35.5 and 35.6 kDa for UUΔ and UAΔ, respectively). pPacUBX Ib$^{ΔN}$ (14) expresses the *UU derivative that lacks UBX residues 37–225.

Cotransfection of Schneider line 2 (S2) cells and CAT assays were performed under our standard conditions (14). Each transfection was done in duplicate, and the results were averaged. Values reported are the average and SE of two or more separate experiments.

## RESULTS

An *Antp* P1-CAT reporter construct that contains P1 sequences from −6 to +0.79 kb was repressed ≈20-fold by both UBX and ABD-A in a cotransfection assay in *Drosophila* S2 cells; ANTP activated the same promoter severalfold (Fig. 1*A*). To localize the cis-regulatory elements required for the differential response to the homeoproteins, deletion derivatives of the P1 construct were tested. A P1 construct was tested that extends from −0.42 to +0.05 kb; it lacks basal enhancer elements that lie upstream of −0.42 but includes a UBX repression element in the core promoter region. This construct was similarly regulated by all three homeoproteins, each repressing expression severalfold (Fig. 1*B*). When either the region upstream of −0.42 (Fig. 1*C*) or the region downstream of +0.05 (Fig. 1*D*) was also present, however, the differential response was restored, with ANTP activating and UBX and ABD-A repressing expression. Thus, either of two regions, one between −6 and −0.42 kb and the other between +0.05 and +0.79 kb, can confer a differential response to the homeotics.

We focus here on the downstream region because it is much smaller and it includes two well-characterized UBX-binding regions that seemed likely to be involved in regulation by the three homeoproteins because they have similar DNA-binding specificities. Furthermore, no P1 sequences upstream of −33 bp are required for the effect of the downstream region (data not shown), and preliminary experiments indicate that the downstream region can confer differential regulation by UBX and ANTP on the *Drosophila Adh* distal promoter (E. Parker and M.A.K., unpublished data). Thus, differential regulation by the homeoproteins does not appear to require any specific elements outside the downstream region.

The two downstream UBX-binding regions, termed A and B here, are located between +290 and +452 bp; each is ≈63 bp long (9). The binding regions are separated by a 37-bp region we call BetBS, for *bet*ween the *b*inding *s*ites (see below). When the binding sites were either deleted (ΔAB, Fig. 2) or replaced with a random sequence (data not shown),
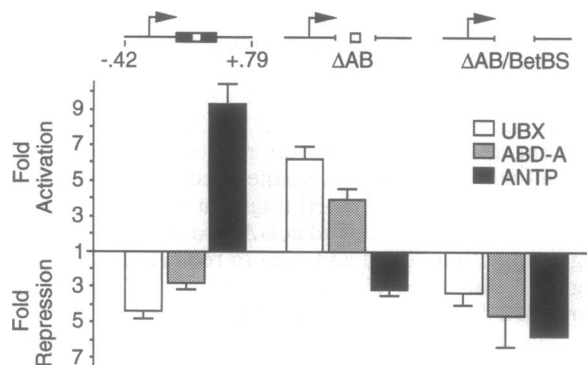
FIG. 2. Homeoprotein regulation of *Antp* P1 constructs lacking the downstream binding sites. Standard cotransfection experiments were done with the UBX, ABD-A, or ANTP expressor plasmids and a promoter construct containing P1 sequences from −0.42 to +0.79 kb, a derivative called ΔAB that lacks UBX-binding regions A and B (filled boxes; each ≈63 bp long; their sequences are given in *Materials and Methods*), or a derivative called ΔAB/BetBS that lacks A and B and the 37-bp region (open box; 5′-CAATCAGTCG-CACACTGTCCATCGCATGGCGCAGATC; called BetBS) that normally separates A and B. The effect of each protein is expressed as fold activation (ratio of promoter activity in the presence of effector protein to that in the presence of a nonexpressing control plasmid) or fold repression (the inverse ratio).

we found that regulation by all three proteins was reversed. ANTP no longer activated P1. Instead, it repressed expression, much as it repressed the P1 construct that contained the central promoter region alone (see Fig. 1B). This result indicates that the downstream UBX-binding sites function as an activation element for ANTP. Consistent with this, the A and B binding regions each conferred dramatic activation by ANTP (≈120-fold and ≈40-fold, respectively) on the *Adh* distal promoter when placed at −47 bp, just upstream of the *Adh* TATA element (data not shown).

In contrast to the results with ANTP, we found that UBX and ABD-A both activated the P1 promoter when the binding sites were removed (ΔAB, Fig. 2). This activation occurs through the BetBS element located between the binding sites: when this 37-bp element was removed along with the A and B binding sites, all three proteins repressed the P1 promoter

(ΔAB/BetBS, Fig. 2). The BetBS element is an unusual UBX and ABD-A activation element, as it is not bound by purified UBX protein; it is described in detail elsewhere (16).

These results establish several important points with respect to homeotic regulatory specificity. (*i*) All three homeoproteins can act through the downstream binding sites. (*ii*) The homeoproteins use the binding sites in very different ways. At their normal position at P1, only ANTP appears to use the binding sites in a simple way, as a conventional activation element. (*iii*) The 37-bp region between the binding sites is itself an unusual regulatory element that discriminates among the homeoproteins: UBX and ABD-A activate through this element, whereas ANTP does not. (*iv*) An interesting interaction exists between the binding sites and the BetBS element, at least for UBX and ABD-A: both proteins appear to act through the binding sites to somehow prevent activation via the neighboring BetBS element. There may also be an analogous effect of neighboring sequences on the function of the homeoprotein bound to the binding sites. We found that UBX activated transcription through the A binding region when it was fused upstream of the *Adh* distal promoter (≈5-fold activation with a single copy and ≈25-fold with two copies in tandem), but this activation function of UBX through the A binding region is apparently suppressed in the context of the *Antp* P1 promoter.

To identify the domains of the homeoproteins responsible for their different regulatory effects, it is reasonable to consider regions that are shared by UBX and ABD-A but different in ANTP. Their primary structures are schematized in Fig. 3. The 60-residue homeodomain region is the only large region of homology, and it is highly similar in all three proteins. Homeodomain residues 2, 24, and 56 are shared by UBX and ABD-A (Arg-2, His-24, and Leu-56) but differ in ANTP (Lys-2, Arg-24, and Trp-56). A short region C-terminal to the homeodomain (filled box in Fig. 3) is also similar among UBX (amino acids AIKELNEQ) and ABD-A (amino acids AVKEINEQ) but different in ANTP (amino acids TKGEPGSG).

To investigate the roles of these regions in regulation, we assayed deletion derivatives of UBX and also chimeric homeoproteins with parts of UBX replaced by ANTP (diagrammed in Fig. 3) for their ability to regulate P1. Fig. 4A shows the effects of the UBX derivatives on the standard P1
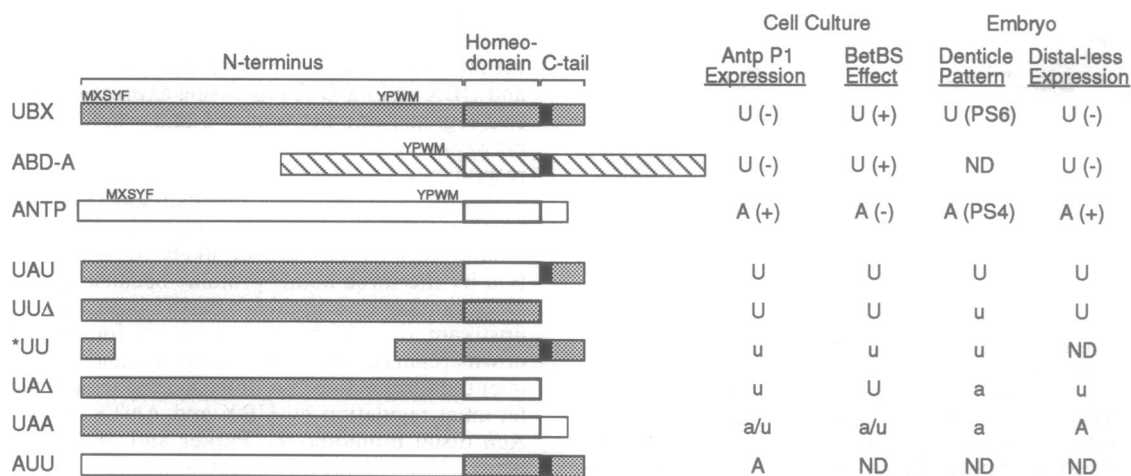


FIG. 3. Comparison of UBX, ABD-A, and ANTP homeoproteins. Diagrams of the primary structures of UBX (stippled box), ABD-A (hatched box), and ANTP (open box) and various UBX–ANTP chimeras and UBX deletion derivatives are shown with a comparison of their effects in four functional assays. Effects in the assays are designated U (UBX-like), A (ANTP-like), u (weak UBX), a (weak ANTP), or a/u (mixed). + and − represent activating or repressing effects, respectively; PS6 refers to the transformation of larval denticle belts to a parasegment 6 identity caused by ectopic expression of UBX, and PS4 refers to the transformation to parasegment 4 identity caused by ectopic expression of ANTP. Protein structures are shown aligned by their homeodomain homology. An 8-residue region of homology between UBX and ABD-A in the C-tail (see text) is shown as a filled box, and other short regions of homology (amino acids MXSYF, YPWM) are also indicated. Cell culture results are from Fig. 4 and embryo results are from ref. 23. Results for AUU are from ref. 10. ND, not determined.
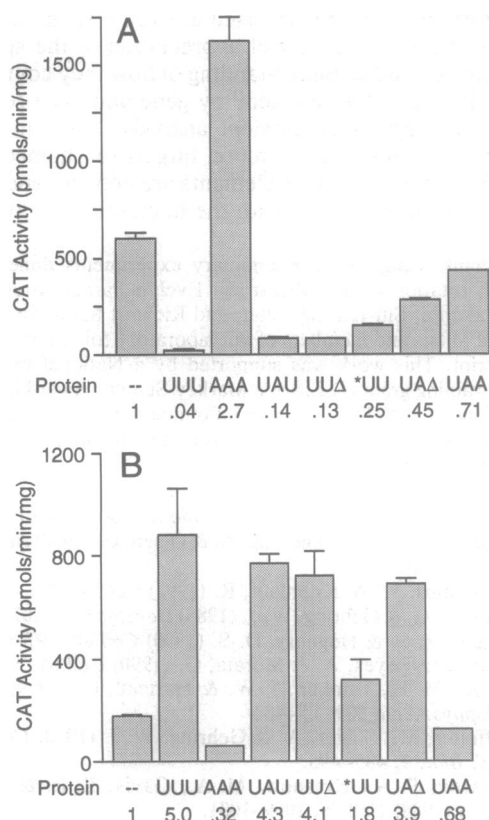
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FIG. 4. Regulation of *Antp* P1 constructs by UBX–ANTP chimeras and UBX deletion derivatives. Effects of the various proteins indicated on the expression of the standard P1 construct (see Fig. 1A) (A) or the ΔAB construct (see Fig. 2) (B) in the cotransfection assay. Relative effects of the proteins are given below each graph. See Fig. 3 for their structures. UUU, UBX Ia protein; AAA, ANTP Ib protein; UAA, UBX N terminus with ANTP homeodomain and C-tail; UAU, UBX with ANTP homeodomain; UUΔ, UBX without C-tail; UAΔ, UBX N terminus with ANTP homeodomain and no C-tail; *UU, large deletion (residues 37–225) of UBX in the N-terminal region. The derivative proteins were expressed at levels similar to that of wild-type UBX Ia (see *Materials and Methods*).

construct, which is repressed by UBX and activated by ANTP (see Fig. 1A), and Fig. 4B shows the effects on a P1 deletion derivative (ΔAB) that lacks the A and B binding regions and is activated by UBX and repressed by ANTP (see Fig. 2). The UBX derivative UAU, which has the UBX homeodomain replaced with that of ANTP, behaved very much like wild-type UBX protein (UUU) in both assays. Elsewhere we have shown that substitutions in the DNA-recognition helix of the UBX homeodomain reduce repression of P1 and eliminate activation through BetBS (16). Taken together, these results indicate that the UBX homeodomain is involved in regulation of P1, but it does not play a critical role in specificity of the response. The UBX derivative that lacks the region C-terminal to the homeodomain (UUΔ) also behaved very much like full-length UBX (Fig. 4), demonstrating that the UBX C-terminal region does not play a crucial role in specificity either. Thus, the two regions common to UBX and ABD-A and absent in ANTP are not the key specificity determinants, although a lesser role for these regions is still possible (see below). The data instead point to an important role for the N-terminal regions. Replacement of the UBX N-terminal region with that of ANTP results in a chimeric protein (AUU) that activated P1 like the original ANTP protein (10), and removal of a large portion of the N-terminal region (*UU) reduced both repression of P1 and activation of the ΔAB construct (Fig. 4).

Although the above results indicate the importance of the N-terminal regions of the ANTP and UBX proteins in dictating regulatory specificity at P1, the other parts of the proteins also appear to contribute. For example, the UAΔ derivative, which contains the intact UBX N-terminal region but has the ANTP homeodomain and no C-terminal region, repressed P1 much less efficiently than UUΔ or wild-type UBX, although it still activated the ΔAB construct like wild-type UBX. Also, the UAA construct had effects that were clearly mixed in the assays, with the ANTP C-terminal region having a particularly significant effect on regulation of the ΔAB construct. This result shows that the ANTP homeodomain and C-terminal region together can partially overcome the effects of the UBX N-terminal region. The converse, however, does not appear to be true, as the AUU construct behaves like ANTP, at least in its regulation of P1 (10).

## DISCUSSION

We have shown that two different regions, located upstream and downstream of the *Antp* P1 promoter, confer differential regulation by the homeoproteins UBX, ABD-A, and ANTP. The downstream region contains two clusters of homeoprotein-binding sites flanking the 37-bp BetBS sequence; it is a composite regulatory element that distinguishes between the homeotics in several ways. The binding sites serve as a conventional activation element for ANTP, whereas the BetBS sequence is an unusual element that is not bound by purified UBX protein but nevertheless can serve as an activation element for UBX and also for ABD-A. UBX and ABD-A both act through the flanking binding sites to prevent activation through the BetBS sequence. These results are summarized in the model in Fig. 5.

Elsewhere we have proposed that action of UBX through BetBS involves an endogenous factor that binds to this region and interacts with UBX (16). We further proposed that UBX bound at the flanking binding sites interacts with the BetBS factors and forms a complex with them that is functionally distinct from the complex formed in the absence of the binding sites. If these ideas are correct, then the results presented here suggest that the interactions with the BetBS factors are specific for UBX and ABD-A. ANTP does not appear to interact with the BetBS factors but instead binds to the flanking sites and presumably interacts with the general
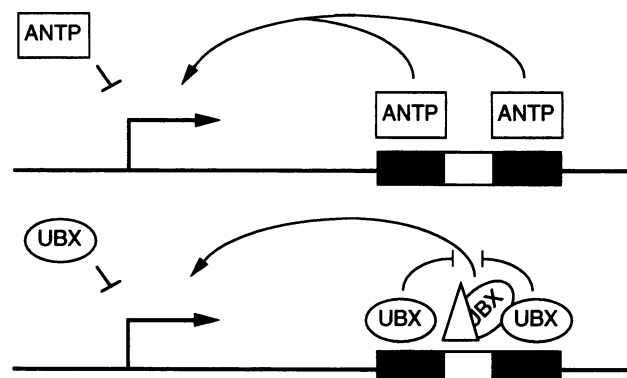


FIG. 5. Model of the differential effects of homeoproteins through the downstream region of *Antp* P1. ANTP activates P1 through the downstream binding sites (■), whereas UBX activates through BetBS (□), perhaps in conjunction with an endogenous factor (△) that binds BetBS. UBX bound at the flanking sites prevents this activation through BetBS. ABD-A behaves as shown for UBX. In addition, all three proteins can repress P1 through the core promoter region, and ANTP can also activate through a separate element (not shown in the diagram) located upstream of −0.42 kb (see Fig. 1).

transcriptional machinery or other factors to activate transcription. Although the specific models are quite speculative, the important conclusion is that UBX, ABD-A, and ANTP each use the downstream binding regions at P1, but UBX and ABD-A use them in a very different manner than ANTP.

Our results demonstrate that differences in activity of the homeotics at a common response element can be critical. However, it seems likely that the overall developmental specificity of the homeotics is achieved through a combination of activity and targeting differences. Although there are few *in vivo* data that bear directly on this issue, recent data suggest that homeotic regulation of the *Antp* P2 promoter in certain neuronal cell types may be governed by activity differences, with ANTP activating through the homeoprotein-binding sites and UBX and ABD-A repressing (13). A number of target genes are known to be regulated by only some homeotics (29), but it is not yet clear that this regulatory specificity is due to targeting differences because the "inactive" homeotics could conceivably target the same regulatory elements but remain functionally silent. One way to assess the relative importance of the two specificity mechanisms *in vivo* is to compare homeoprotein-binding sites on polytene chromosomes (29). If different chromosomal regions are bound by the various homeoproteins, then an important role for targeting is indicated, but if all the same regions are bound, then activity differences are probably key.

The results presented here and elsewhere (10, 14) demonstrate the importance of the N-terminal domain, and lesser roles for the homeodomain and the C-terminal region, in dictating homeodomain regulatory specificity at the *Antp* P1 promoter in S2 cells. This is surprising for two reasons. (*i*) While UBX and ABD-A appear to exert similar effects through the same regulatory elements at P1, there are no sequence homologies in their N-terminal domains that are not also shared by ANTP. This observation implies that the common functional specificity of UBX and ABD-A derives from unrelated structures in the two proteins or from similarities that will only be apparent in their folded structures. (*ii*) Most studies of homeoprotein specificity have found that the critical specificity determinants lie in the homeodomain and possibly the surrounding sequences (20–22, 24), whereas our results point to a more important role for regions outside the homeodomain, particularly the N-terminal region. Our results are generally in accord with those of Chan and Mann (23), who analyzed the effect of many of the same chimeras on denticle patterning and repression of the Distal-less gene during embryonic development (summarized in Fig. 3). However, there are differences. The most notable is that the UAΔ chimera behaved like UBX with respect to *Antp* P1 repression in our assays and also with respect to Distal-less repression in the embryos, whereas its effect on denticle patterning in the embryo was more like that of ANTP. Also, the UAA construct acted like ANTP with respect to Distal-less regulation but had mixed effects on *Antp* P1 expression. The most reasonable explanation of these differences is that there are multiple structural determinants of homeoprotein functional specificity, with different structural domains important for protein and DNA interactions at different regulatory elements. Whereas the *in vivo* phenotypic assays provide an aggregate measure of the effects on many target genes in many tissues, our work has shown that multiple

regulatory mechanisms are used at even a single target in a single cell type. Thus, a full appreciation of the specificity determinants and an understanding of how they contribute to specificity may require a gene by gene and even regulatory element by regulatory element analysis. Such an analysis may become feasible as more targets of homeotics are identified, their regulatory elements are defined, and some of the proteins that interact with the homeotics are known.

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